Interleukin-12 Primes Human CD4 and CD8 T Cell Clones for High Production of Both Interferon-γ and Interleukin-10

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Summary

Interleukin-12 (IL-12) induces differentiation of T helper 1 (Th1) cells, primarily through its ability to prime T cells for high interferon-γ (IFN-γ) production. We now report that the presence of IL-12 during the first several days of in vitro clonal expansion in limiting dilution cultures of polyclonally stimulated human peripheral blood $CD4^+$ and $CD8^+$ T cells also induces stable priming for high IL-10 production. This effect was demonstrated with T cells from both healthy donors and HIV(+) patients. Priming for IL-4 production, which requires IL-4, was maximum in cultures containing both IL-12 and IL-4. IL-4 modestly inhibited the IL-12-induced priming for IFN-γ, but almost completely suppressed the priming for IL-10 production. A proportion of the clones generated from memory $CD45RO^+$ cells, but not those generated from naive $CD45RO^-$ $CD4^+$ T cells, produced some combinations of IFN-γ, IL-10, and IL-4 even in the absence of IL-12 and IL-4, suggesting in vivo cytokine priming; virtually all $CD4^+$ clones generated from either CD45RO(-) or (+) cells, however, produced high levels of both IFN-γ and IL-10 when IL-12 was present during expansion. These results indicate that each Th1-type (IFN-γ) and Th2-type (IL-4 and IL-10) cytokine gene is independently regulated in human T cells and that the dichotomy between T cells with the cytokine production pattern of Th1 and Th2 cells is not due to a direct differentiation-inducing effect of immunoregulatory cytokines, but rather to secondary selective mechanisms. Particular combinations of cytokines induce a predominant generation of T cell clones with anomalous patterns of cytokine production (e.g., IFN-γ and IL-4 or IFN-γ and IL-10) that can also be found in a proportion of fresh peripheral blood T cells with "memory" phenotype or clones generated from them and that may identify novel Th subsets with immunoregulatory functions.

The immune response to infectious agents and to nominal antigens is often characterized by a dominance of either cell-mediated or humoral-type effector mechanisms (1), which has been attributed to a dichotomy in the cytokine production pattern of T helper (Th) $CD4^+$ cells (2). Two classes of Th cells have been described: Th1 cells, which produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin (LT) and favor cell-mediated immunity, delayed-type hypersensitivity, macrophage activation, and production of opsonizing antibodies; and Th2 cells, which produce IL-4, IL-5, IL-6, IL-10, and favor humoral responses, production of IgE and IgA, and activation of eosinophils and basophils. The differentiation of Th cells toward a Th1 or Th2 phenotype occurs early during an immune response and is influenced by many interrelated factors, including the nature and the concentration of the antigen, the anatomical localization of the immune response, the nature of the antigen-presenting cells (APC), and the cytokine milieu at the site of the immune response (3). IL-4, produced by T cell subsets and possibly basophils, appears to be the most important cytokine responsible for Th2 cell generation (4), whereas IL-12, produced by macrophages and professional APC such as dendritic cells and Langerhans cells (5–8), is required for effective Th1 cell generation (9–12). IL-12 acts at three different levels in favoring a Th1 response: (*a*) it acts as a proinflammatory cytokine inducing IFN-γ from T and NK cells within a few hours after infection, and through IFN-γ it induces activation of phagocytic cells for bacteriocidal activity and also for enhanced production of cytokines, including IL-12 itself (6, 13–15); (*b*) in the first few days of an immune response, IL-12 and

Drs. Gerosa and Paganin contributed equally to this work.

IL-12-induced IFN-γ act on antigen-stimulated T cells to favor differentiation and proliferation of cells with a Th1 phenotype (9–12); (*c*) once differentiated Th1 are generated, IL-12 is required for optimal cytokine production and, in certain cases, proliferation of Th1 cells (16). One of the central mechanisms by which IL-12 induces differentiation of Th1 cells is its ability to prime T cells during clonal expansion for high IFN-γ production (17–19). This priming effect of IL-12 is stable, and T cell clones originally expanded in the presence of IL-12 maintain the ability to produce high IFN-γ levels upon restimulation even when cultured for at least several weeks in vitro in the absence of IL-12 (17). However, IL-12 when present during restimulation also enhances IFN-γ production from established Th1 clones (16, 17) and transiently induces low level IFN-γ production from established Th2 clones (17, 20). Like $CD4^+$ T cells, $CD8^+$ T cells also display either a Th1 or Th2 phenotype (17, 21, 22), and IL-12 induces a priming for high IFN-γ production in both $CD4^+$ and $CD8^+$ T cells (17, 18).

Although the expression of Th1-type and Th2-type cytokines was initially considered to be mutually exclusive, Th cells expressing both Th1 and Th2 cytokines (referred to as Th0 cells) have been identified both during differentiation of Th cells (23) and in terminally differentiated cells (24). Indeed, evidence for a stochastic acquisition of cytokine profile, suggesting independent regulation of each cytokine gene, was obtained during a mouse Th2 response to keyhole limpet hemocyanin (KLH) (25) and in human peripheral blood memory $CD4^+$ T cells (26, 27). Our findings that IL-12 has a potent priming effect on human T cells for high IFN-γ production, but no direct effect on IL-4 production by T cell clones, also strongly suggested an independent regulation of IFN-γ and IL-4 gene expression (17). IL-10, like IL-4, was thought to be produced exclusively by Th2 cells in the mouse (2), but it has since been reported that both mouse and human IL-10-producing cells can coexpress either IL-4 or IFN-γ (28, 29).

In this paper, we analyzed the effect of IL-12 on the expression of IL-10 in human $CD4^+$ and $CD8^+$ T cell clones. We find that IL-12 when present in the limiting dilution cultures in the first few days of clonal expansion, primes $CD4^+$ and $CD8^+$ T cell clones for high production of both IFN-γ and IL-10. IL-12 does not prime for IL-4 production, which requires the presence of IL-4 during clonal expansion; however, priming for IL-4 production is maximal in cultures containing both IL-12 and IL-4. IL-4 has a modest inhibitory effect on the IL-12-induced priming for IFN-γ production, but almost completely suppresses the priming for IL-10 production.

Materials and Methods

Reagents. Phytohemagglutinin (PHA) was purchased from Grand Island Biological Co. (Grand Island, NY). *Dermatophagoides pteronyssinus* group I (Der p I) was kindly provided from Lofarma Allergeni (Milan, Italy), and 12-0-tetradecanoyl-phorbol-13-acetate (TPA)[1] was from Sigma Chemical Co. (St. Louis, MO). Anti-CD3, anti-CD4, and anti-CD8 mAbs were purchased from Ortho Pharmaceuticals (Raritan, NJ) and Becton-Dickinson (Mountain View, CA), or produced from cells obtained from American Type Culture Collection (ATCC, Rockville, MD). Human recombinant IL-2 (rIL-2) was a gift of Eurocetus (Milan, Italy) and of Dr. T. Taguchi (Osaka University, Osaka, Japan). Human rIL-12 (CHO-cell derived) was provided by Dr. Stan Wolf (Genetics Institute Inc., Cambridge, MA). Human rIL-4 was purchased from Genzyme (Cambridge, MA) and had a specific activity of 10^7 U/mg. Human rIFN-γ (7×10^7 U/mg) was provided by Dr. H.M. Shepard (Genentech Inc., South San Francisco, CA).

Subjects. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque gradient separation from peripheral blood of healthy volunteers and human immunodeficiency virus type 1 (HIV-1)-infected individuals, recruited from the Immunodeficiency Program of the Hospital of the University of Pennsylvania. $CD4^+$ lymphocyte counts were obtained on the same day that patients' blood was obtained for use in this study. Patients were categorized according to the Centers for Disease Control criteria on the basis of $CD4^+$ lymphocyte number and HIV-related symptomatology and complications (30).

Generation of $CD4^+$ and $CD8^+$ T Cell Clones. T cell clones were generated from PBMC by limiting dilution (17, 31). 0.5 PBMC/well were seeded in 96-well round-bottom tissue culture plates in 200 μl of RPMI-1640 medium with 10% FCS in the presence of 5 μg/ml PHA, γ-irradiated (50 Gy) autologous (for cloning of healthy donor T cells) or allogeneic (for cloning of HIV(+) patients' T cells) PBMC (2.5×10^4 cell/well) and RPMI-8866 B lymphoblastoid cells (10^4 cells/well) in the presence of IL-12 (2.5 ng/ml), IL-4 (50 U/ml), neutralizing anti-IL-12 mAb C8.6 (ascites, 1:400, sufficient to completely neutralize more than 10 ng/ml of IL-12, a level higher than that possibly produced by the feeder cells) (6), anti-IL-4 neutralizing antibodies 4F2 and 5A4 (32), a combination of cytokines and antibodies or culture medium. In some experiments, limiting dilution cloning was performed in U-bottom plates coated with anti-CD3 mAb (OKT3, 5 μg/ml in carbonate buffer, pH 9.5) instead of in the presence of PHA. After 3 days, IL-2 (50 U/ml, final concentration) was added to all cultures by replenishing half of the medium. After 1 wk, irradiated PBMC (5×10^5/well) and RPMI-8866 (10^4/well) as feeder cells were added again; cells were maintained in culture with IL-2 and fed and split as required with the original combination of antibodies and/or cytokines maintained through the culture period, unless otherwise indicated. Cloning efficiency was calculated for $CD4^+$ or $CD8^+$ by Poisson distribution as described (17, 31, 33); only wells containing a sufficient T cell number to allow determination of both surface phenotype and cytokine profile were considered positive. The proportion of $CD4^+$ and $CD8^+$ T cells in the plated PBMC was taken into account in calculating the number of cells from either subset plated per well.

Surface Phenotype of T Cells. The phenotype of PBMC and T cell clones was examined by immunofluorescence (flow cytometry) using FITC-conjugated anti-CD3, anti-CD4, and anti-CD8 mAbs as described (17).

Sorting of $CD45RO^+$ and RO^- T Cells. PBMC were stained by indirect immunofluorescence using the anti-CD45RO mAb Leu 45RO (Becton Dickinson) and FITC goat anti-mouse Ig (Becton Dickinson). Cells were sorted using a Coulter Elite cell

[1] *Abbreviation used in this paper:* TPA, 12-0-tetradecanoyl-phorbol-13-acetate.

sorter (Hialeah, FL). Before sorting, 37% of lymphocytes were $CD45RO^+$. After sorting, purified $CD45RO^-$ cells were contaminated with 1.2% $CD45RO^+$ cells and purified $CD45RO^+$ cells were contaminated with 2% $CD45RO^-$ cells. Immediately after sorting, T cells were cloned by limiting dilution, as described above.

Induction of Cytokine Production by T Cell Clones. To induce cytokine production, T cell clones were resuspended in complete medium to a concentration of approximately 10^6/ml and stimulated for 24 h with soluble anti-CD3 mAb (OKT3, ascites 1:10,000) and TPA (10 ng/ml). Cell-free culture supernatants were collected and stored in aliquots at −70°C until used.

Quantitation of IFN-γ, IL-4, and IL-10. IFN-γ was quantitated in T cell clone supernatants by radioimmunoassay (RIA) using mAbs B133.1 and B133.5 (34); IL-4 was quantitated by RIA using antibodies 4F2 and 5A4 (32), kindly provided by Dr. Lucien Aarden (Central Laboratory of the Netherlands Red Cross, Amsterdam); IL-10 was quantitated by RIA using antibodies JE53 9D7 and 12G8, kindly provided by Dr. Anne O'Garra (DNAX, Palo Alto, CA). Recombinant human IFN-γ, IL-4, and IL-10 were used as reference standards.

Statistical Analysis. The statistical significance for the difference in cytokine production by the group of clones cultured in the different conditions was evaluated by student's *t* test; significant and not significant, when not specifically indicated, refer to $P < 0.05$ and $P > 0.05$, respectively.

Results

IL-12 Primes $CD4^+$ and $CD8^+$ Clones for IL-10 Production and IL-4 Inhibits Such Priming. Freshly isolated peripheral blood lymphocytes (PBL) from two donors were cloned by limiting dilution in the presence of feeder cells, IL-2, and PHA or anti-CD3 antibodies bound to plastic. IL-12 and/or IL-4 was also added to some of the plates. When IL-12 or IL-4 was not added, the respective neutralizing antibodies were added to block endogenous cytokines. After 4 wk of culture, clones were washed, stimulated with soluble anti-CD3 antibodies and TPA for 18 h, and cytokine production in the supernatant was evaluated by RIA (Fig. 1). As previously reported (17–19), the presence of IL-12 during cloning induced a several-fold increase in the ability of both $CD4^+$ and $CD8^+$ clones to produce IFN-γ, whereas it had a little and non-significant effect on IL-4 production. The addition of IL-4, either alone or with IL-12, had no significant effect on the production of IL-4, but partially inhibited the priming for IFN-γ production (Fig. 1). The failure in these experiments to identify a requirement for IL-4 in the generation of IL-4 producing cells was likely due to the presence within the unfractionated PBL or memory T cells which, as described below, generate clones with high and variable ability to produce IL-4 even when expanded in the absence of IL-4. In addition to priming the T cell clones for IFN-γ production, IL-12, present during cloning, also primed them for high IL-10 production, an effect observed in both cloning conditions (PHA or anti-CD3) and with both $CD4^+$ and $CD8^+$ clones (Fig. 1). IL-4 alone had no significant effect on the ability of the clones to produce IL-10, but significantly and almost completely inhibited the priming effect of IL-12 on IL-10 production.

The ability of IL-12 or anti-IL-12 antibodies present during limiting dilution cloning to prime $CD4^+$ and $CD8^+$ clones for IL-10 production was also observed in PBL from five other healthy individuals and 10 HIV(+) patients at various stages of the disease; analysis of all $CD4^+$ and all $CD8^+$ clones from either healthy donors (Fig. 2 *A*) or HIV(+) patients (Fig. 2 *B*) revealed significant IL-10 production after IL-12 priming ($P = 0.027$ for $CD4^+$ cells from HIV(+) patients; $P \leq 0.001$ in all other groups). In several of the experiments represented in Fig. 2, IL-12 was present only during the first 2 wk of the 4-wk cloning period.

Clones Derived from Naive and Memory $CD4^+$ T Cells Are Differentially Affected by IL-12. Because of the high variability in cytokine production by clones derived from total PBL, even when cultured in the absence of added cytokines, we separately cloned $CD4^+$ cells with a memory ($CD45RO^+$) or naive phenotype ($CD45RO^-$) (35) (Figs. 3 and 4). The large majority of $CD4^+$ clones generated from $CD45RO^-$ cells in the presence of neutralizing antibodies to IL-12 and IL-4 produced negligible amounts of IFN-γ, IL-10, or IL-4 (Fig. 4). By contrast, approximately half of the clones derived from $CD45RO^+$ cells produced IFN-γ and IL-10, even when expanded in the presence of anti-IL-12 antibodies, and IL-4 even when expanded in the presence of anti-IL-4 (Fig. 3). When cultured in the presence of IL-12, all clones produced high levels of IFN-γ and IL-10, and when cultured in the presence of IL-4, most although not all the clones produced high levels of IL-4. However, the differential ability of the clones to produce the various cytokines depending on the culture conditions was much more obvious in the clones derived from $CD45RO^-$ cells (Figs. 3 and 4). These clones expanded in the presence of anti-IL-12 produced very low levels of IFN-γ or IL-10, which on average were increased 125-fold and 29-fold, respectively, in the clones expanded in the presence of IL-12. The production of IL-10 by the different clones was more variable than that of IFN-γ, as clearly shown by the SE in Fig. 4. Thus, whereas IL-12 was an absolute requirement for high production of IFN-γ, a minor proportion of clones produced moderate to high IL-10 even when cultured in the absence of IL-12. IL-4 was required for priming $CD45RO^-$ cells for IL-4 production, but endogenously produced IL-4 in the cultures (most likely by the irradiated feeder cells) was sufficient, because addition of neutralizing antibodies to IL-4 to the cultures blocked IL-4 production by the clones, whereas addition of exogenous IL-4 only modestly increased IL-4 production as compared to that by clones generated without either IL-4 or anti-IL-4 antibodies (Fig. 4). Interestingly, the highest production of IL-4 was reproducibly observed in clones generated in the presence of both IL-12 and IL-4, although all these clones also produced high levels of IFN-γ. In the presence of added IL-4, but not IL-12, approximately a quarter of the clones have a Th2 phenotype (i.e., they produced moderate to high amounts of both IL-4 and IL-10, but not IFN-γ). The presence of IL-4 during the expansion of the clones, as with total PBL cloning (Fig. 1), modestly decreased the priming effect of IL-12 for IFN-γ production, but almost

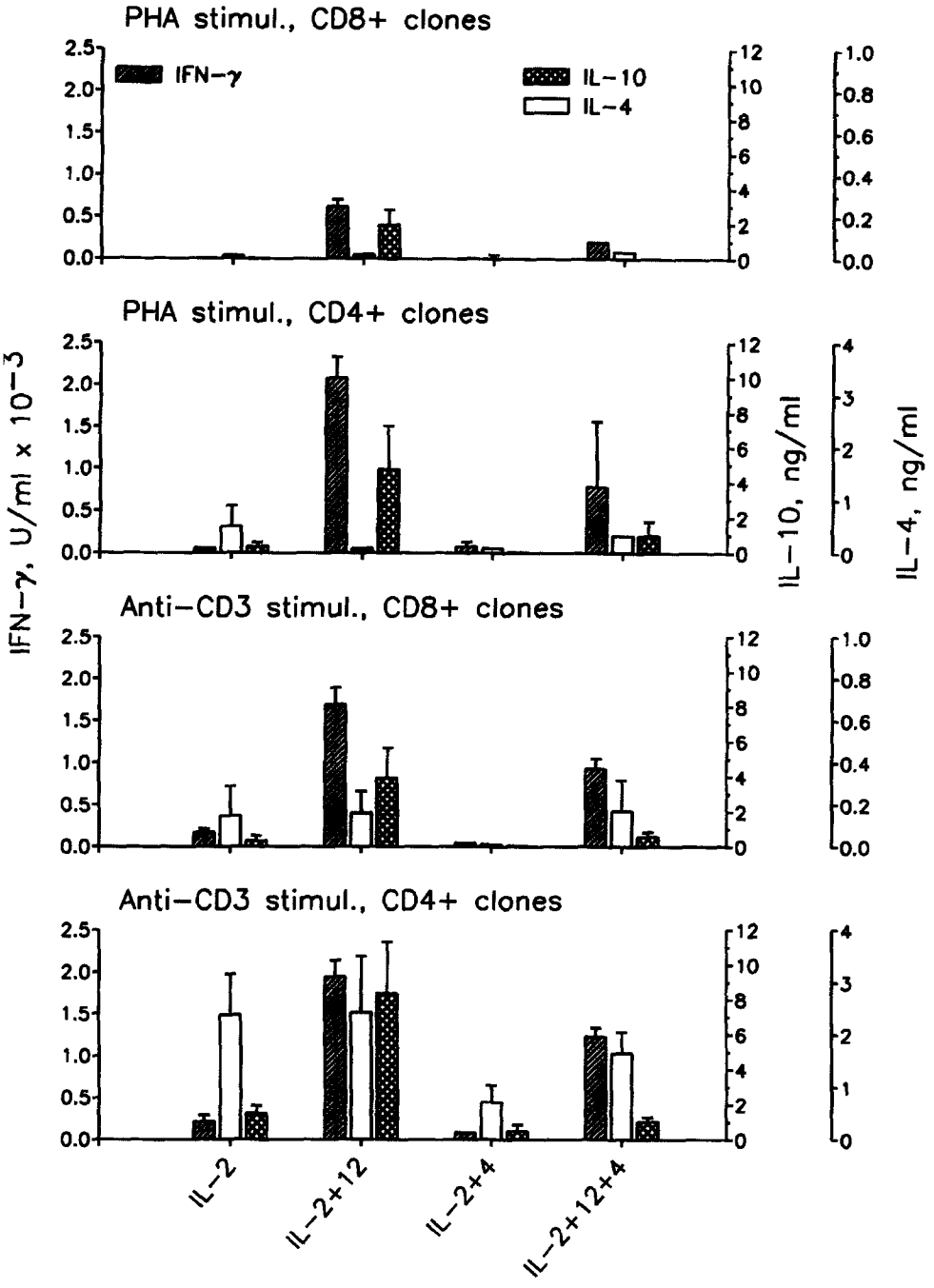

Figure 1. IL-12 primes $CD4^+$ and $CD8^+$ T cell clones for production of IFN-γ and IL-10. PBL from one donor were cloned in the presence of PHA and from another donor in the presence of plastic-bound anti-CD3 antibodies, and the clones were expanded in the presence of feeder cells (irradiated autologous PBMC and RPMI-8866 cell line) and the indicated cytokines for 4 wk. IL-2 was used at 50 U/ml, IL-12 at 2.5 ng/ml, and IL-4 at 50 U/ml. When IL-12 or IL-4 was not added, the respective neutralizing antibodies were used to block endogenous cytokines. Clonal growth efficiency was on average ~50%, and lower (20–30%) when anti-IL-4 was present. Clones were then washed in medium without cytokines, stimulated for 18 h with soluble anti-CD3 antibodies and TPA as described in Experimental Procedures, and cytokine production in the supernatant was quantitated by RIA. Data represents mean ± SE of all clones analyzed.

completely suppressed the IL-12 priming effect for IL-10 production (Fig. 4).

The Priming Effect of IL-12 for IL-10 and IFN-γ Production Is Not Mediated by IFN-γ. We investigated whether IFN-γ induced by IL-12 plays a role in the priming of the T cell clones for IFN-γ and IL-10 production and whether IFN-γ could replace IL-12 in inducing such a priming. As shown in Table 1, neutralizing anti-IFN-γ antibodies did not prevent the IL-12-induced priming for IFN-γ production in clones originated from either $CD4^+$ $CD45RO^-$ or $CD8^+$ $CD45RO^-$ T cells, and exogenous recombinant IFN-γ added to the cultures in the absence of IL-12, could not induce priming. In the case of priming for IL-10 production, anti-IFN-γ antibodies partially but significantly ($P < 0.05$) decreased it in $CD4^+$ T cell clones, but have no effect in $CD8^+$ T cell clones; in both types of clones, IFN-γ could not replace IL-12 to induce IL-10 priming. No significant difference was observed in all these experimental conditions for IL-4 production by the clones.

The Priming Effect of IL-12 for IL-10 Production Is Stable and Occurs in the First Few Days of Clonal Expansion. The priming effect of IL-12 on IL-10 production is stable, because

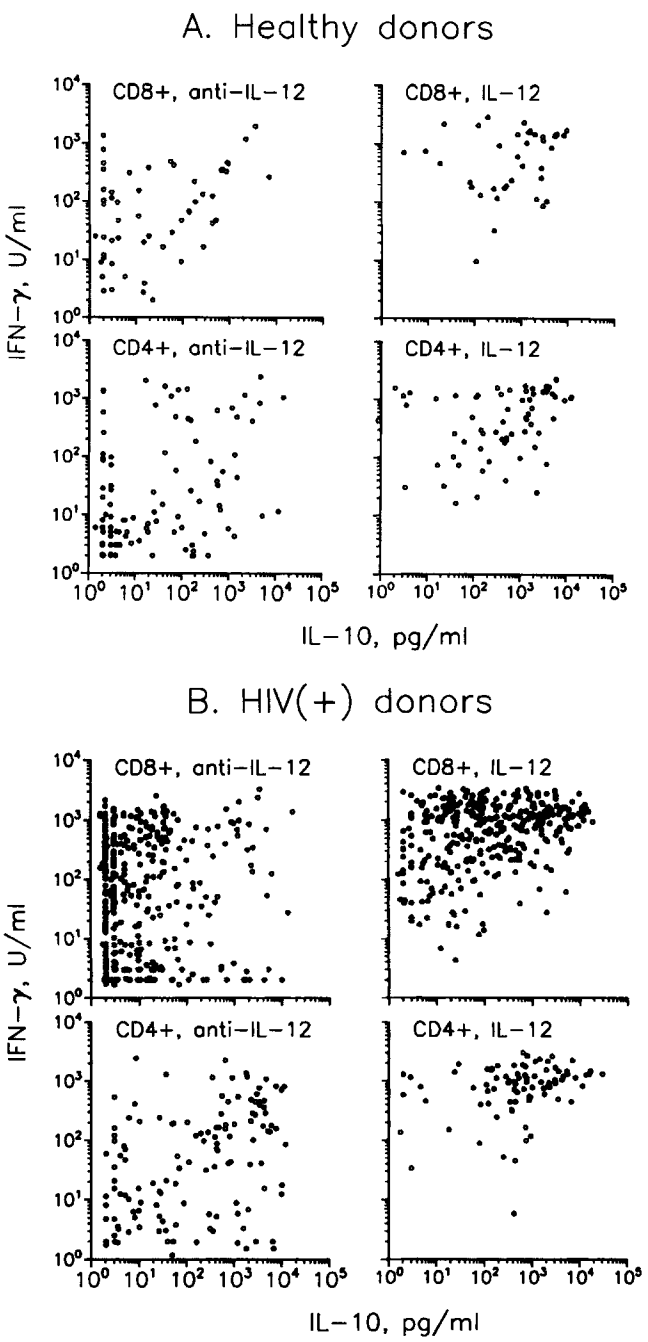

Figure 2. Production of IFN-γ and IL-10 by $CD4^+$ and $CD8^+$ clones from 7 different healthy patients (*A*) and 10 HIV(+) patients (*B*) generated by limiting dilution in the presence of neutralizing anti-IL-12 antibodies (C8.6, ascites, 1:400) or recombinant human IL-12 (2.5 ng/ml). The PBMC used as irradiated feeder cells were autologous for the cloning of healthy donors' PBL and from allogeneic healthy donors for the cloning of HIV(+) patients' PBL. For several of the donors, IL-12 was added only in the first 2 wk of cultures. After 5 weeks of culture, the $CD4^+$ (bottom) and $CD8^+$ clones (top) were washed and stimulated with anti-CD3 and TPA for 18 h. Cytokines were measured in the cell-free supernatant fluids by RIA. Each symbol represents the cytokine production by one individual clone. The results in *A* are cumulative of the 7 healthy donors; the results in *B* are from the 10 HIV(+) patients. IL-12 treatment significantly increased the ability of the clones to produce IFN-γ ($P < 0.001$) and IL-10 ($P = 0.027$ for $CD4^+$ cells from HIV(+) patients, $P \leq 0.001$ in all other groups).

addition of neutralizing anti-IL-12 antibodies after 1 wk of limiting dilution culture in the presence of IL-12 only partially decreased and after 2 wk did not affect the priming for both IFN-γ and IL-10 production (results not shown). To determine when during clonal expansion IL-12 induces priming for IFN-γ and IL-10 production, low levels of neutralizing anti-IL-12 antibodies were added at the time of limiting dilution to prevent the effect of endogenously produced IL-12, and IL-12 at concentrations able to overcome the effect of the neutralizing antibodies was added at different times of culture. The priming effect was observed when IL-12 was added at day 0 or 3, but not when added at day 7 or later (Fig. 5).

Discussion

Accumulating evidence from in vivo and in vitro experimental systems supports the conclusion that IL-12 not only promotes Th1-type immune responses, but is also required in vivo for optimal Th1 responses (8–10, 36). In all these experimental systems, IL-12 not only induced an increase in IFN-γ production, but also very efficiently suppressed the production of IL-4. However, the in vivo studies or the in vitro analysis based on the effect of IL-12 on bulk polyclonal T cell cultures did not distinguish between a direct effect of IL-12 on the differentiation of the Th clones, or an indirect effect favoring preferential expansion of Th1 cells or inhibition of Th2 cell growth. To distinguish between direct effects on Th cell differentiation and selective effects, we (17) analyzed the effect of IL-12 on the cytokine production of human $CD4^+$ and $CD8^+$ T cells in a limiting dilution cloning system which allows the clonal expansion of almost every single T cell from peripheral blood. Because of the high clonal efficiency and the seeding of less than one cell per well, this experimental system allowed us to test direct differentiative effects of IL-12 at the clonal level. Whereas IL-12 induced a powerful priming for high IFN-γ production in every single clone analyzed, it had no significant effect on the ability of the clones to produce IL-4 (17). Thus, it was concluded that the priming for high IFN-γ production is a direct differentiation-inducing effect of IL-12, while the inhibition of IL-4 production may involve preferential expansion of Th1 cells, due perhaps to the lack of functional IL-12 receptors on Th2 cells (37, 38), or a selective growth inhibition of Th2 cells by IFN-γ (39), most likely due to the downmodulation of the IFN-γ receptor β chain in Th1 cells (38, 40).

There is relatively little information on the effect of IL-12 on T cell production of IL-10, but studies with bulk T cell cultures from atopic patients stimulated in vitro with allergen have shown that production of both Th2 cytokines, IL-4 and IL-10, is inhibited if IL-12 is added to the cultures (41). We have been able to reproduce these results both in bulk cultures or analyzing antigen-specific clones derived from these cultures (our unpublished results). However, in vivo treatment with IL-12 has been reported to result in a dramatic increase in both IFN-γ mRNA and IL-10 mRNA

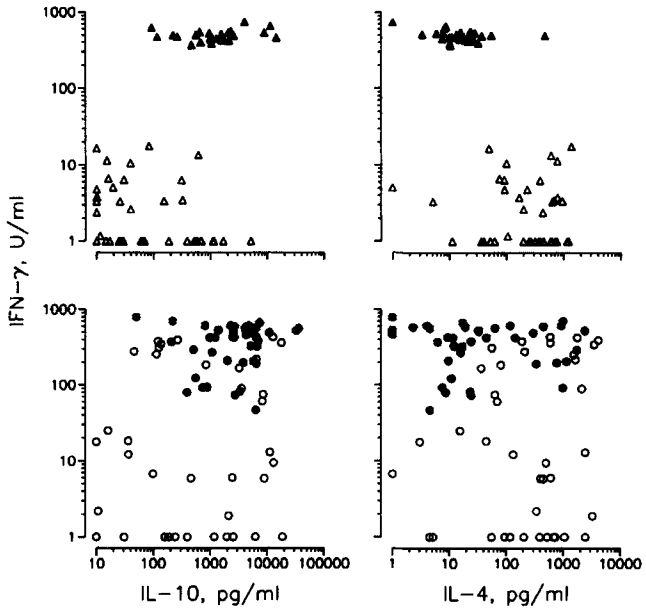

Figure 3. Cytokine production by CD4+ clones generated from CD45RO− and CD45RO+ T cells. PBL were sorted into CD45RO− and RO+ subsets and cloned by limiting dilution in the presence of anti-IL-12 antibody (C8.6, ascites, 1:400) and human recombinant IL-4 (50 U/ml) or human recombinant IL-12 (2.5 ng/ml) and anti-IL-4 antibodies (4F2 and 5A4, ascites, 1:100). After 4 wk, clones were washed, incubated for 24 h in medium containing only IL-2 (50 U/ml), washed again, and stimulated with anti-CD3 and TPA; the cell-free supernatant fluid was collected after 18-h stimulation and cytokines were quantitated by RIA. Each dot represents a single CD4+ clone. This experiment is representative of three performed with superimposable results. △, CD45RO(−), IL-4, anti-IL-12; ▲, CD45RO(−), IL-12, anti-IL-4; ○, CD45RO(+), IL-4, anti-IL-12; ●, CD45RO(+), IL-12, anti-IL-4.

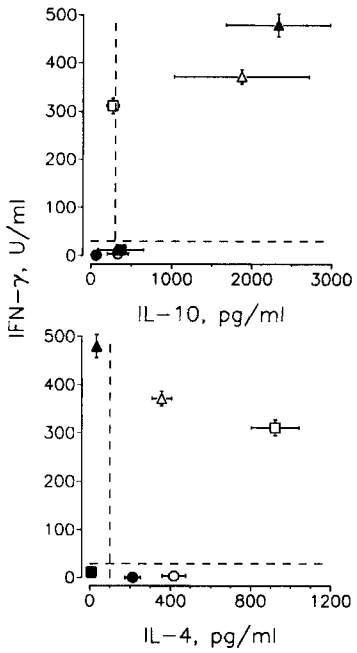

Figure 4. Cytokine production by CD4+ clones generated from sorted CD45RO− cells. CD45RO− cells were cloned by limiting dilution (PHA stimulation) in the presence or absence of IL-12 (2.5 ng/ml), IL-4 (50 U/ml), anti-IL-12 neutralizing mAb (C8.6, ascites, 1:400), anti-IL-4 neutralizing mAb (4F2 and 5A4, ascites, 1:100), or combinations thereof. After 4 wk, the clones were washed, incubated for 24 h in medium containing IL-2 (50 U/ml) only, washed again, and then stimulated for 18 h with soluble anti-CD3 antibodies and TPA; cytokines were measured in the cell-free supernatant fluid by RIA. The graphs report the mean ± SE of the cytokine production of all clones analyzed in each cloning condition. Arbitrary threshold of cytokine production (broken lines) are drawn to facilitate the analysis of the results. Clonal efficiency was ~50%, except that in the cultures in the presence of anti-IL-4, where the efficiency was 20–30%. Data from this experiment are representative of three cloning experiments with similar results, except the cloning in the presence of both anti-IL-4 and anti-IL-12, which generated a sufficient number of clones for analysis in only two experiments. ■, (anti-IL-12 + anti-IL-4) (n = 11); ●, (anti-IL-12) (n = 36); ▲, IL-12 (anti-IL-4) (n = 26); △, IL-12 (n = 43); □, IL-12 + IL-4 (n = 51); ○, IL-4 (anti-IL-12) (n = 41).

(42), although the nature of the cells accumulating IL-10 mRNA, either T cells or macrophages, was not investigated.

Our present data show that the presence of IL-12 in vitro during limiting dilution cloning of human CD4+ or CD8+ T cells results in an efficient priming of the clones for high production of both IFN-γ and IL-10. This result was observed with both anti-CD3 or PHA stimulation and with PBL from both healthy donors and HIV(+) patients, from early to very advanced stages of HIV disease. These findings are consistent with the observation that T cells from HIV patients are fully responsive to IL-12 (18, 43, 44), although their phagocytic cells and APC might be defective in IL-12 production (45, 46).

The high variability in production of cytokines observed in human CD4+ T cell clones expanded in the absence of exogenous IL-12 and IL-4 or in the presence of neutralizing antibodies against these two cytokines suggests that some of the cells were already primed in vivo for cytokine production. The cloning of sorted CD45RO− naive CD4+ cells and CD45RO+ memory CD4+ cells supported this interpretation. A high proportion of the clones generated in the presence of neutralizing antibodies to IL-12 and IL-4 produced one or a combination of IFN-γ, IL-4 and IL-10, with a pattern of production that was not always consistent with the classical paradigm of Th1 and Th2 cells. When CD45RO− cells were cloned in the same conditions, the clones produced only negligible amounts of the three cytokines. However, in both populations, the presence of IL-12 during cloning endowed virtually all clones with the ability to produce high levels of IFN-γ and IL-10. These data confirm our previous observations that IL-12 can induce a high IFN-γ-producing Th1 phenotype not only in naive T cells, but also in memory T cells, in response to either polyclonal activation or specific recall antigens (9, 17). Surprisingly, the same observation held true for the priming of IL-10 production in a limiting dilution condition.

Added IL-4 or endogenously produced IL-4 was necessary in the limiting dilution culture to prime T cell clones generated from CD45RO− cells for IL-4 production, whereas approximately half of the clones generated from CD45RO+ cells produced IL-4 even when expanded in the absence of IL-4. Because of this high variability in IL-4 production in memory T cells, the requirement for IL-4 in the generation

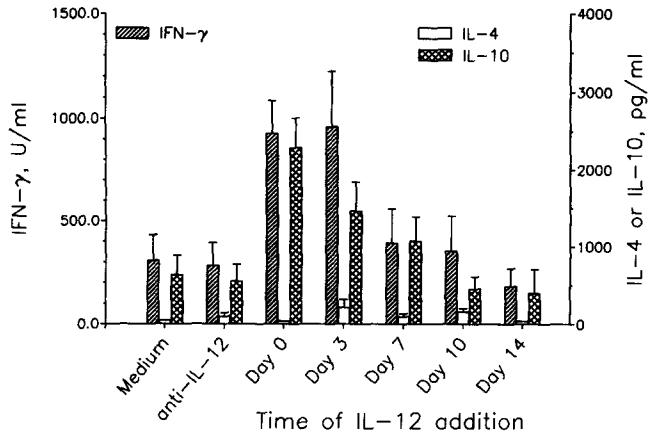

Figure 5. IL-12 induces priming for high IFN-γ and IL-10 production in $CD4^+$ clones only when added during the first few days of clonal expansion. PBL from normal donors were cloned by limiting dilution (PHA stimulation) in the presence of medium or neutralizing anti-IL-12 antibodies (C8.6, ascites, 1:1,000); in different plates, IL-12 (5 ng/ml) was then added at day 0, 3, 7, 10, and 14 after limiting dilution cloning. This concentration of IL-12 overcame the inhibitory effect of the low concentrations of anti-IL-12 antibodies, as determined empirically. After 5 wk, clones were washed and restimulated with soluble anti-CD3 and TPA for 18 h. Cytokines were measured by RIA in cell-free supernatant fluids. Bars show mean + SE of all clones analyzed in each condition of culture. This experiment is representative of three cloning experiments with superimposable results for both $CD4^+$ and $CD8^+$ clones.

of IL-4 producing cells could not be demonstrated in $CD4^+$ $CD45RO^+$ cells (Fig. 3) or unfractionated PBL (Fig. 1). Although IL-12 is a major and probably necessary inducer of a Th1 response, in several experimental conditions, it also potentiated IL-4 production and the development of Th2 cells from naive $CD4^+$ murine T cells (47) and from neonatal $CD4^+$ human T cells (48), and potentiated a Th2 response to *Schistosoma mansoni* in IFN-γ knockout mice (49). Our present data show that IL-12 does not prevent IL-4 production from $CD4^+$ clones derived from limiting dilutions of naive adult peripheral blood $CD45RO^-$ cells and, in fact, significantly enhances the ability of IL-4 to prime the clones for high IL-4 production, thus extending the previously published results (47–49) by demonstrating that IL-12 enhances IL-4 production at the single clonal level via a differentiation effect. Furthermore, when T cells were cloned in the simultaneous presence of IL-12 and IL-4, the IFN-γ priming effect of IL-12 was only partially and often not significantly inhibited by IL-4, whereas the priming for IL-10 production was reproducibly and almost completely blocked by IL-4. Thus, paradoxically, IL-4 is more potent in inhibiting priming of Th cells for production of a Th2-type cytokine than for the typical Th1-type cytokine, IFN-γ.

Several authors have reported the requirement of IFN-γ production for the induction of Th1 response in vivo and in vitro (11, 49–52); however, this role for IFN-γ was not confirmed in other murine studies (53, 54) and in none of the human studies (9, 41, 48). In the present study, neutralizing anti-IFN-γ antibodies did not prevent the IL-12-induced priming for IFN-γ production and only partially decreased priming for IL-10 in $CD4^+$ but not in $CD8^+$ clones. Recombinant IFN-γ could not replace IL-12 in the priming for either IFN-γ or IL-10 production.

We have previously shown that the priming for high IFN-γ-induced production induced by IL-12 is stable and that if IL-12 is present in the first 2 wk of culture, the clones can be cultured for at least 3 more weeks in the absence of IL-12 and maintain their ability to produce high levels of IFN-γ (17, 18). For induction of IFN-γ production in the clones, IL-12 needs not be present during stim-

Table 1. *Role of IFN-γ in IL-12-mediated Priming of Human $CD4^+$ and $CD8^+$ T Cell Clones for IFN-γ and IL-10 Production*

Cells*	Cloning conditions‡	Number of clones	IFN-γ	IL-10	IL-4
			U/ml	*pg/ml*	*pg/ml*
$CD4^+$ $CD45RO^-$	anti-IL-12	17	132 ± 57§	122 ± 98	25 ± 7
	anti-IL-12 + IFN-γ	12	58 ± 34	12 ± 7	36 ± 11
	IL-12	13	374 ± 39	3413 ± 1094	99 ± 16
	IL-12 + anti-IFN-γ	13	332 ± 43	853 ± 243	72 ± 29
$CD8^+$ $CD45RO^-$	anti-IL-12	9	126 ± 75	37 ± 19	6 ± 2
	anti-Il-12 + IFN-γ	15	161 ± 50	120 ± 33	13 ± 6
	IL-12	23	476 ± 20	957 ± 395	11 ± 3
	IL-12 + anti-IFN-γ	22	430 ± 18	1909 ± 462	9 ± 2

*Sorted $CD4^+$ $CD45RO^-$ and $CD8^+$ $CD45RO^-$ peripheral blood lymphocytes were cloned by limiting dilution (PHA stimulation) in the presence of the indicated antibodies or cytokines.

‡T cell clones were expanded for 4 wk in the presence of neutralizing anti-IL-12 mAb (C8.6 ascites, 1:400), anti-IL-12 mAb and recombinant human IFN-γ (1,000 U/ml), IL-12 (2–5 ng/ml), or IL-12 and neutralizing anti-IFN-γ mAb (B133.3 ascites, 1:400) as described in Materials and Methods.

§After being expanded for 4 wk, the indicated number of clones was stimulated with anti-CD3 mAb and TPA and production of the indicated cytokine was measured; results are mean ± SE of all clones analyzed. The results are representative of two experiments with similar data.

ulation with various IFN-γ inducers; however, when present, it increases IFN-γ production, both in IFN-γ producer clones and, at lower levels, in non-IFN-γ producer Th2 clones (17, 20). We now find that the priming for high IL-10 production induced by IL-12 has very similar characteristics to that of IFN-γ production and that IL-10 priming is induced when IL-12 is present in the limiting dilution culture in the first 1 or 2 wk and is maintained when cells are cultured in the absence of IL-12. As with IFN-γ, the presence of IL-12 is not required during stimulation of the clones for IL-10 production, but its presence enhances IL-10 production. The time of addition of IL-12 to the limiting dilution cultures is also critical: if IL-12 is added at day 0 or 3, priming for both IFN-γ and IL-10 is observed; however, if added at day 7 or later, almost no priming effect is observed. These results suggest that the clones are susceptible to the priming effect of IL-12 in a very narrow temporal window during the first few days of clonal expansion.

IL-12 primes T cells for IFN-γ and IL-10 production in a similar fashion, but its ability to induce acute expression of either cytokine is quite different. In freshly isolated PBL, IL-12 alone induces IFN-γ production and synergizes with many other stimuli (14), whereas in the same conditions, no induction of IL-10 is observed, although a more detailed analysis of different stimulatory conditions remains to be performed. However, a few days of culture of PBL stimulated with PHA or anti-CD3 in the presence of IL-12 results in high constitutive expression of IL-10, and IL-12 strongly potentiates the acute production of IL-10 observed in $CD4^+$ and $CD8^+$ T cell clones stimulated with anti-CD3 antibodies (D. Peritt and G. Trinchieri, manuscript in preparation). Although the mechanism of this priming remains elusive, it is clearly distinct from that responsible for acute IFN-γ and IL-10 production, which is still effective on advanced or terminally differentiated Th1 and Th2 clones (17, 20), and is reminiscent of the commitment to a Th1 or Th2 response in vivo that is observed within a few days after immunization (11, 25).

In conclusion, our present results and previous reports indicate that IL-12 induces priming for high IFN-γ (17) and IL-10 production (this article). We also confirm in our clonal assay with human T cells that IL-4 is required for IL-4 production priming (4). IL-12 and IL-4 modulate each other's effect, i.e., IL-4 acting at the clonal level partially decreases the priming for high IFN-γ production induced by IL-12 and very efficiently prevents the priming for IL-10 production. At the clonal level, the ability of IL-12 to inhibit IL-4 production, a striking phenomenon in vivo or in polyclonal cultures, is not observed and instead IL-12 enhances the ability of IL-4 to prime for IL-4 production. Thus, similar to recent observations in the mouse system (25, 55), our results suggest that the IFN-γ, IL-4, and IL-10 cytokine genes are independently regulated and that any combination of these three cytokines can be observed in clones generated from human peripheral blood without a clear distinction along the Th1 or Th2 phenotype, even when the priming effect of IL-12 or IL-4 in vitro is neutralized using specific antibodies. However, when antigen-specific clones are generated in vivo or in vitro in polyclonal cultures, a preferential clustering of Th1 or Th2-type cytokines is observed, both at the polyclonal and the clonal level. Because IL-12 and IL-4 do not induce this dichotomy at the single cell (or clonal) level in vitro, it is likely that the mechanisms leading to the Th1/Th2 polarization involve secondary induced factors and/or selective mechanisms, which may be influenced by the nature and concentration of the antigen, the APC, and other cytokines present at the site of the immune response. However, it is striking that many of the clones obtained from memory human T cells cultured in the absence of IL-12 and IL-4 (a condition that does not endow naive CD4 cells with the ability to produce cytokines) can produce high levels of one or more of the cytokines IFN-γ, IL-4, and IL-10. The pattern of production of these three cytokines appears to be stochastic, with no clear polarization of the cells to a Th1 or Th2-type phenotype. Similarly, coexpression of IFN-γ and IL-4 was described in a proportion of memory $CD4^+$ T cells with the $CD45RO^+$ or $CD27^-$ phenotype (26, 27). Because $CD4^+$ T cells in those studies were analyzed immediately after purification from peripheral blood, possible artifacts due to the long-term cloning cultures were excluded. Note that like the studies analyzing cytokine production in freshly isolated $CD4^+$ peripheral blood when actively stimulated (26, 27), our work shows that clones generated from $CD4^+$ $CD45RO^-$ cells in the absence of IL-12 or IL-4 are unable to produce cytokines, whereas clones generated from $CD4^+$ $CD45RO^+$ cells produce cytokines with a pattern reflecting that of freshly isolated $CD45RO^+$ cells. The cloning procedure in the presence of both IL-2 and a polyclonal stimulator, such as PHA, does not alter the cytokine profile of the $CD4^+$ cells, thus representing a powerful method to study the role of cytokines such as IL-12 during clonal expansion in determining the cytokine profile of responding $CD4^+$ T cells.

The Th2 cytokine IL-10 has a profound inhibitory effect on the ability of phagocytic cells to produce IL-12 (56), and this effect may represent one of the mechanisms by which a Th1 response is downregulated when Th2-type cells are activated. However, this simple interpretation is now complicated by the observation that $CD4^+$ cells producing both IFN-γ and IL-10 exist in humans and in the mouse (28, 29), and can easily be cloned from peripheral blood T cells (this study and 57). Human T cell clones producing IFN-γ and IL-10 have an anti-inflammatory function, inhibiting macrophage activation and T cell proliferation (57), and can be isolated with high frequency from T cell populations within the rheumatoid synovial membrane (58), where they may be a major contributor to the endogenous immunosuppression that occurs in rheumatoid arthritis. Notably high IFN-γ- and IL-10-producing clones are induced by stimulation with an antigenic peptide of proteolipoprotein, which is mutated in the major TCR-interacting residue and which protects mice from allergic encephalitis induced by the wild-type peptide; these IFN-γ- and IL-10-producing clones have an anti-encephalitogenic effect in vivo, suggesting that they might represent cell types

involved in the downregulation of Th1 responses (59). The $CD4^+$ cell types with unusual cytokine production profiles may represent new subsets of Th cells, with effector functions different from the typical Th1 and Th2 cells and mediating different effects on specific aspects of humoral and/or cellular immune response. An alternative interpretation is that successful immune responses are characterized by a polarized Th1 or Th2 response, whereas ineffective responses resulting in anergy or tolerization may be characterized by anomalous cytokine production phenotypes that fail to sustain an effective cellular or humoral immune response. The accumulation of these cell types in the memory compartment of peripheral blood $CD4^+$ cells may represent the end product of unsuccessful or repressed immune responses. In particular, the extreme phenotype of high IFN-γ and IL-10 production induced by IL-12 may represent a cell type with a downregulatory effect on Th1 responses, by suppressing expression of IL-12, TNF-α, IL-1β, B7, and other costimulatory molecules on APC as well as proliferation of Th1 cells (56, 60).

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